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HORIZONTAL GENE TRANSFER IN PLASMID: ARE WE CLOSE TO ELIMINATING PERIODONTAL PATHOGENS?

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ABSTRACT

In clinical periodontal practice, patients with periodontitis frequently yield subgingival pathogens, resistant to therapeutic concentrations of commonly used antibiotics. The oral cavity is believed to provide excellent exogenous horizontal DNA transfer in dental plaque bacteria. Plasmids harness the replication, transcription, and translation systems of their host to ensure their continuity. While doing so, they burden their hosts' functionality. The non-specificity and inefficiency of current antibiotics and plasmid-mediated drug resistance is a major emerging challenge in dentistry.

The literature reports that Actinobacllus actinomycetemcomitans, Fusobacterium nucleatum, Campylobacter, Streptococcus sp., and other periodontal pathogens are known to harbor native plasmids. The elimination of plasmids involves the inhibition of its replication in the "rolling circle" model. To the best of our knowledge, this paper is the first to discuss plasmid prevalence in periodontal microbiota. Furthermore, it also sheds light on plausible plasmid, curing that depends on 1) the chemical structure of curing compounds, 2) entry into the bacterial cell membrane, 3) prevention of the covalent binding of the Rep protein to the interns and Pcr A helices, and 4) inhibition of DNA gyrase might result in cessation of R-plasmid replication. By hindering plasmids rolling circular replication, it might be possible to reduce the spread of antimicrobial resistance genes and some virulence factors.

KEY WORDS: Plasmid, Curing, Replication & Periodontitis

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Original Article

INTRODUCTION

Antimicrobial chemotherapy is frequently employed in combination with surgery, curettage, or alone to treat patients with refractory periodontitis and various extra oral infections (Van Winkelhoff et al. 1996). It is effective at first, but eventually, there is growing bacterial resistance to many of the antibiotics, therefore limiting their use in periodontal disease. This resistance increases 105 types of bacterial genetic availabilities, a phenomenon described as "genetic capitalism" (Fernando et al. 2011). Data from the literature suggest that antibiotic resistance is found to be increased in periodontal biofilms (Roberts et al. 2010). However, there is a scarcity of information on bacterial species isolated from patients with periodontitis, since microbial sensitivity tests are not normally performed in daily practice of dentistry (Geisla Mary et al. 2012).

An increase in the emergence of multi-drug resistant bacteria in recent years is associated with increased use of available higher generation of antibiotics. It is clinically a challenging condition, as it predisposes patients to the risk of developing uncontrolled life-threatening infections. This review article focuses on the following aspects: Horizontal gene Transfer (HGT) mechanism, plasmid prevalence, mechanism of replication inhibition and curing in periodontal bacteria.

Mechanism of Horizontal Gene Transfer in Bacteria

The oral cavity can act as a reservoir of antibiotic-resistant microorganisms, some of which are capable of causing local and systemic diseases. A few of such bacteria contain multiple virulence genes called 'pathogenicity islands' that are located on large and unstable regions of the bacterial genome. These pathogenicity islands can be transmitted to other bacteria by HGT, thereby causing changes in the bacterial genome. It can occur among bacteria in the dental plaque, where they remain in close and stable proximity during colonization (Rajiv et al. 2011). Biofilm-associated periodontal bacteria communicate with each other more easily by way of HGT (Tatakis et al. 2005). Recent and bioinformatics studies have confirmed that HGT is a common occurrence in human microbiota (Liu et al. 2012). Currently, there are three known mechanisms by which HGT occurs (Gogarten & Townsend 2005): (1) transformation, (2) transduction and (3) conjugation.

In Transformation, lateral transfer of mobile genetic elements between diverse bacteria leads to a rapid dissemination of genes encoding resistance to antibiotics. Periodontal bacteria, including members of genus *Streptococcus, Neisseria and Actinobacillus,* are naturally competent and have specialized systems for DNA uptake (Rajiv et al. 2011). Transformation by extracellular DNA has been shown to mediate the transfer of antibiotic resistance in an oral biofilm model (Hannan et al. 2010). *Streptococcus mutans* from biofilm has transformation efficiency that was at least 10- to 600-fold higher than in planktonic *S. mutans* cells (Li et al. 2001). Interestingly, DNA release and transformation seem to be part of the biofilm-related life cycle, and the released DNA has capacity to stabilize biofilm structure and architecture (Whitchurch et al. 2002).

Transduction, similar to transformation except that, the exogenous bacterial DNA carrying resistance determinant is transferred by a phage particle (Ammann et al. (2008). Studies on saliva have isolated bacteriophages with a) bactericidal effect against specific bacterial pathogens such as *Enterococcus faecalis,* for example (Stevens et al. 2009) and b) transduction property capable of transferring antibiotic resistance genes. The example includes temperate bacteriophage that transfers genes among *Actinobacillus actinomycetemcomitans* strains (Sandmeier et al. 1995, Roy 2015).

Conjugation: Bacterial conjugation is believed to be the most important mechanism responsible for HGT (Liu et al. 2012). Resistance to antibiotics in pathogenic organisms often resides in extra-chromosomal DNA (plasmid). Plasmids are able to replicate independently from the normal chromosomal DNA, through a process called conjugation. This was first discovered in Japan in the 1950s in the gastroenteritis-causing *Shigella dysenteriae* and identified the causative factor be resistance plasmid (R-plasmid). Such plasmids acts as a key vehicle, and have proved to be a great threat to the medical science, as it is quicker than native mutation and vertical evolution (Jafari et al. 2013). Multiple drug resistance plasmid develops as a result of the integration of two DNA segments with different drug resistance property, as a single double-stranded circular molecule (Figure 1).

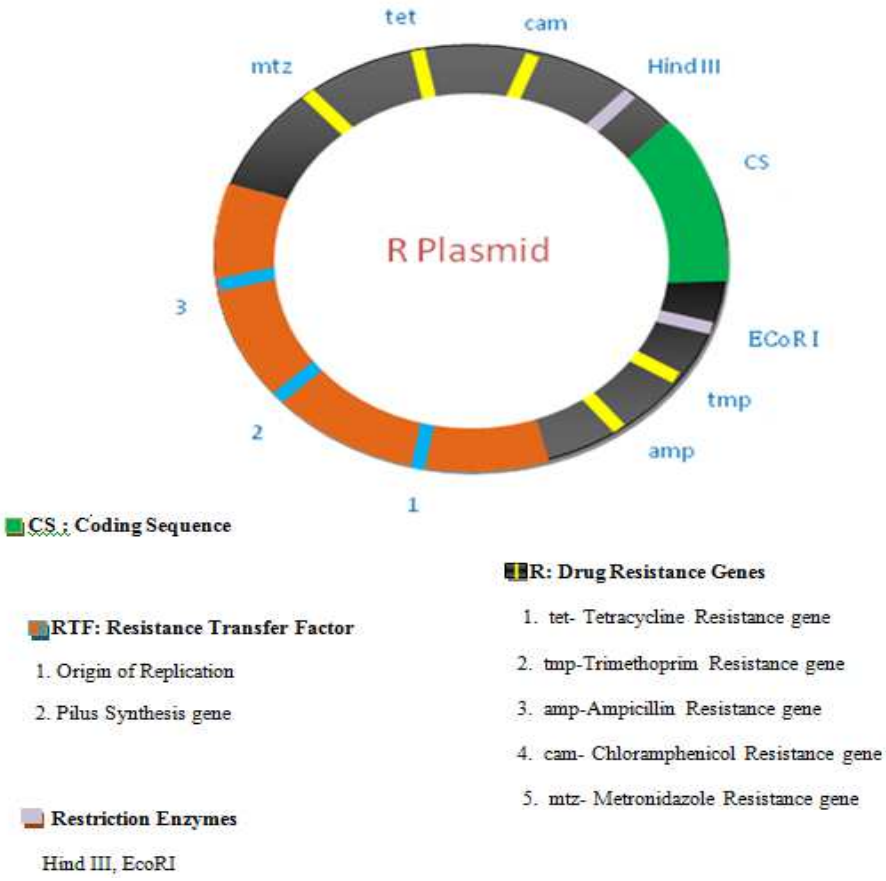

Figure 1: Diagrammatic Representation of R- Plasmid Consisting of Two Segments, RTF and Resistance Determining Segment

One DNA segment contains resistance transfer factor (RTF), while the other segment contains the drug-resistance genes. The RTF is mainly involved in the transfer function of the R-plasmid and contains a number of transfer genes, including those controlling replication of the plasmid in the host cell. Hence R- plasmids carry a considerable variety of genes that code for functions other than transfer and replication, such as antibiotic resistance and resistance to a number of toxic heavy metals, metabolic enzyme, adhesion, and bacteriocin production (Gouby 1986). The resistance genes located in the other segment elaborate enzymes for the destruction of the antibacterial drugs, like the penicillins, streptomycin, chloramphenicol, tetracyclines, kanamycin, sulfonamides etc. For example, tetracyclines have been used in the treatment of various periodontitis (Palmer 1996). The gene that codes for tetracycline in R-plasmid inactivates the antibiotic and represent a cooperative behavior, as the entire bacterial population benefits of removing the antibiotic (Slots & Rosling 1983). The high density of cells in biofilms increases the spread of antibiotic resistance plasmids by conjugation, and conjugation itself may stimulate biofilm formation within the plaque population in periodontitis (Rita et al. 2011). However, the very nature of their importance to the antibiotic resistant phenotype may expose R-plasmid s as the Achilles' heel of drug-resistant bacteria**.**

Rolling Circular Replication in Plasmid

Rolling circular (RC) plasmids were originally discovered in *S. aureus* and have now been described in a large

number of periodontal pathogens such as *A. actinomycetemcomitans* (Dominique et al. 2001) and *S. mutans* (Anke et al. 2013). Such rolling replication occurs in bacterial plasmids that contain antibiotic-resistance genes and are used to transfer plasmid between bacteria. Several detailed reviews have been published dealing with plasmid RC replication (Cesar et al. 2016, Gruss & Ehrlich 1989). A supercoiled plasmid is first nicked by a RepC dimer. The double-stranded origin facilitates binding and/or nicking of the DNA by RepC. PcrA helicase interacts with RepC and unwinds the duplex DNA. Then, a series of concerted cleavage/joining reactions occur that result in the release of a circular, leading-strand as DNA and a nicked open-circular DNA containing the newly replicated leading strand. The neck is then sealed by the host DNA ligase, and the DNA is subsequently converted to the supercoiled form with DNA gyrase. Projan and Novick (1988) noted that, the Rep proteins that are highly involved in RC replication are encoded by antibiotic resistance plasmids of the staphylococcal family, and in diversified bacterial species gain the resistance longevity, because of this replication function.

Why Seek for Plasmid Curing?

As the problem of antibiotic resistance grows, and the number of new antibiotics fails to provide a solution, novel approaches are required to tackle the issue. The very fact that plasmids are responsible for large swaths of drug-resistance in bacteria makes them attractive antibacterial targets. Thomas et al. (2005) exploited this natural mechanism in the identification of small molecule mimics of plasmid incompatibility, “anti-plasmid” compounds that eliminate plasmids from the bacterial population and re-sensitize the bacteria to antibiotics. Apparently, the ideal way to minimize plasmid transmission of antibiotic resistance is to eliminate, or “cure” them from their hosts are of great clinical significance. Many compounds have been shown to be capable of causing this effect [26]. Curing may occur naturally through cell division or by exposing the host to chemical and physical agents (Letchumanan et al. 2010), such as elevated temperatures, intercalating dyes (acridine orange, ethidium bromide), crystal violet, sodium dodecyl sulfate (SDS), thymidine starvation and exposure to UV radiation. Different plasmids vary considerably in their property to be cured. Some curing agents work in a non-specific way by damaging and stressing out the cells, while some seem to act much more selectively (Zaman et al. 2010). However, as shown by Lujan and coworkers (2007), the prevention of plasmid replication can indeed directly induce cell death, a surprising and welcome discovery.

Plasmid Curing in Periodontal Condition

There are not many reports on plasmid prevalence and curing in patients with periodontitis. HGT by plasmid is thought to play a crucial role in the evolution of *Fusobacteria* species. The presence of plasmids observed in oral *F. nucleatum* strains isolated from periodontal patients and its absence in healthy subjects suggested an association with some pathogenic factors. *S. mutans* is one of the common initiators of dental caries. Distribution of plasmid DNA within a mother-child population significantly clustered in mother-child pairs compared with unrelated individuals (Caufield et al. 1988). *Streptococcus sp.* had their ability to serve as donors of the erythromycin and lincomycin resistance marker plasmid (Donald et al. 1978). The prevalence of plasmid in oral pathogen *A. actinomycetemcomitans* might help in equipping them with a variety of determinants in plasmids, such as virulence and drug resistance (Mario et al. 2001). Below is the summary of various reports regarding the plasmid curing status in aerobic and anaerobic periodontal pathogens from patients with different ailments like respiratory tract infections, wound infections, urinary tract infections, skin burns, exudative specimens (pus, saliva, wound swabs, ear swabs and body fluids), blood and urine (Table 1a and 1b).

Table 1a: List of the Plasmid Curing Status of Anaerobic Periodontal Bacteria

ORGANISM	CURING AGENT	CONCENTRATION	MEDIA	PLASMID SIZE	OBSERVATION	REFERENCE
Anaerobic bacteria (Gram+ve) *Bifidobacterium pseudolongum*	Arabinose, Fructose, Galactose and Sucrose	0.15%	Nutrient Broth	-	PC	Stefano T et al., 2012
Actinobacillus actinomycetemcomitans	-	-	-	Three plasmids (30,70,90 kb)	-	Olsvik B et al., 1989
Streptococcus mutans	*Morus alba* extract	25 µg	Brain Heart Infusion Broth	5.6 kb	PC	Smitha C & Usha R 2016
Enterococcus faecalis	*Morus alba* extract	25 µg	Brain Heart Infusion Broth	5 to150 kb	PC	Smitha C & Usha R 2016
Streptococcus intermedius	EtBr	0.5 µg	Mueller Hinton Agar	2.65 kb	PC	Bjorland J et al., 2003 [36]
Streptococcus simulans	EtBr	0.5 µg	Mueller Hinton Agar	2.65 kb	PC	Bjorland J et al., 2003
Streptococcus viridans	SDS	10%	Nutrient Broth		PC	Akortha EE et al., 2011
Fusobacterium nucleatum	-	-	Blood Agar	6.2 kb	PC	Abdulkareem AA 2009
(Gram –ve) *Eikenella corrodens*	AO	-	-	8.7 kb	PC	Hiroyuki A et al., 2006
Capnocytophaga ochracea	EtBr	-	Mueller Hinton Broth	9 kb	PC	Agnes R et al., 2000
Camphylobacter jejuni	EtBr,AO, Acriflavine, Rifampin, ⇧ temperature	160 µg Rifampin 3.2 µg EtBr 12.5 µg AO 12.5 µg Acriflavine	Luria Bertani Broth	≥21 kb	Rifampin showed curing effect	Dasmeh H et al., 2015

Table 1b: List of the Plasmid Curing Status of Aerobic Periodontal Bacteria

ORGANISM	CURING AGENT	CONCENTRATION	MEDIA	PLASMID SIZE	OBSERVATION	REFERENCE
Aerobic bacteria (Gram +ve) *Staphylococcus aureus*	AO	0.1mg	Nutrient Broth	23 kb	PC	Chaudhari K et al., 2015
	AO	0.10mg	Mueller Hinton Broth	-	PC	Ojo SKS et al., 2014
	AO and SDS	0.25 mg AO 1g SDS	Nutrient Broth	11 to18 kb	PC	*Charles OE* et al., 2010
	SDS	10%	Nutrient Broth	-	PC	Akortha EE et al., 2011
Lactobacillus acidophilus	Morus Alba Extract	25 µg	Brain Heart Infusion Broth	-	PC	Smitha C & Usha R 2016
(Gram –ve) *Haemophilus parainfluenzae*	Mitomycin C	0.2 µg	Brain Heart Infusion Broth	5.25 kb	PC	Helen MW et al., 1991
Escherichia coli	AO and SDS	0.25 mg AO,1g SDS	Nutrient Broth	11to18 kb	PC	*Charles OE* et al., 2010
Acinetobacter baumannii	AO and ⇧ Temperature	0.1mg	Nutrient Agar	-	No PC⇧ temperature. PC with AO	Dasmeh H et al., 2015
	AO and EtBr	640 -2560 µg AO 320 -5120 µg EtBr	Luria Bertani Broth	500 bp to ≥ 25 kb	PC	Saranathan R et al., 2014
	EtBr, AO	300 -400 µg EtBr 300- 400 µg AO	Luria Bertani Broth	3.5 kb to 10.0 kb	No PC AO, PC with EtBr	Pahwa S et al., 2012
Pseudomonas aeruginosa	SDS, EtBr and Temperature (45°C)	700 µg EtBr 1% SDS	Nutrient Broth	20 to 100 Mda	PC	Hayfaa ME et al., 2013, Shinji T et al., 1984
Klebsiella pneumonia	AO And SDS	0.25 mg AO 1g SDS	Nutrient Broth	11 to 18 kb	PC	*Charles OE* et al., 2010
	SDS	10%	Nutrient Broth	-	PC	Akortha EE et al., 2011
Serratia marcescens	AO	200 µg	Luria Bertani Broth	-	PC	Nageswaran N et al., 2012
	L-Ascorbic acid	50%	Nutrient Broth	-	PC	Wameidh M P et al., 2009
	Ciprofloxacin	0.4 mg	Nutrient Broth	-	PC	Llanes C et al., 1989

Campylobacter plasmids were detected in 60% *C. jejuni*, 50% *C. coli* and 80% *C. lari* isolates. Most of the

Camphylobacter sp. possessing chloromphenicol resistant marker were plasmid-mediated (Baserisalehi et al 2004). *Staphylococcal* antibiotic resistance was associated with resistant BlaZ gene in the plasmid that had the ability to mediate the production of drug-inactivating enzymes, such as β-lactamase (Daini & Akano 2009). Because of this location, the gene is easily movable to surrounding cells through plasmid (Diep 2006). According to Spengler et al. (2006) curing agents, including ethidium bromide acted on plasmid either via inhibition of plasmid efflux pumps on the plasmid membrane or inhibition of DNA gyrase responsible for plasmid DNA replication. R-plasmids were not only maintained stable, but also transferred between bacterial cells at a very high efficiency, in many cases approaching 100%. Earlier, it was noted that EtBr curing led to the disappearance of resistance to an extended spectrum of β-lactams (ESBL) with the concurrent loss of plasmids from *A. baumannii*, suggesting that, ESBL determinants were plasmid-borne. Plasmids were randomly distributed in *E. coli* isolates, where plasmid curing converted resistant isolates into susceptible form (if resistance is plasmid-mediated) (Sekimizu & Kornberg 1988). In *Enterobacteriaceae*, (Sharon et al, 1995) noted that plasmid encoding *CTX-M* and *TEM* enzymes had greatly influenced the resistance of these species to antibiotics, globally.

Plasmid Curing Mechanism

Knowledge of mechanisms, which can specifically hinder plasmid replication phenomena is an important tool for a microbial geneticist to overcome the antimicrobial resistance in prevailing pathogens.

Involvement of Chemical Structure of Curing Agents

Spengler et al. (2006) reported that the amphiphilic compounds have a planar ring system by substitution in the L-molecular region. This could keep apart the super helical form of plasmid DNA in forming an open circle, or linear plasmid DNA to disturb plasmid replication process, with an increase in melting point and circular dichroism. So, it can be inferred that the anti-plasmid action of the curing compounds depend on its chemical structure with a planar ring system.

Involvement of Cell Membrane Protein DNA

For replication, some plasmids require membrane binding for initiation and active participation of plasmid. The host integral membrane protein DnaA, associated with the membrane of *E. coli in vivo* is also required by many plasmids such as RK2 plasmid for example, for initiation (Farshad et al. 2012). A strong correlation also exists between the association of the TrfA initiation protein of plasmid in the binding of plasmid origin RK2 replication *in vitro* or plasmid DNA *in vivo* (Saleem et al. 1997). The curing agent sodium dodecyl sulfate produced its curing effect after penetrating through the cell membrane, and disrupted plasmid DNA replication by interfering with genes responsible for resistance or inactivation of necessary enzymes (Stephen et al. 2015). It is interesting to note that the DnaA is required by many plasmids for initiation and separation of AT-rich regions in the plasmid origin for elongation (Iordanescu 1993).

Thus, our review suggests that the plasmid curing molecules could bind to host integral cell membrane protein DnaA, by blocking the AT-rich separation in elongation, and further might inactivate the recognition of TrfA or similar TrfA like initiation proteins of the plasmid, to hinder the plasmid replication. Thus, in our opinion, the question is not whether replication and partitioning are membrane associated, but how they are interrelated in *in vivo.*

Involvement of Repc Protein and PcrA Helicase

The replication and copy number of RC plasmids, in general, are regulated at the level of synthesis of their Rep proteins [64] and its homology codes for many staphylococcal resistance plasmids (Christie 2014). Another protein PcrA, a

DNA helicase involved in unwinding plasmids, which carry antibiotic resistance genes and initiates replication and repair DNA damage (Marie-Agnès et al. 1998).

We propose that the curing agents could cause plasmid DNA to "buckle" and inactivation of the covalent binding of the Rep protein to the iterations of the hairpin loop of DNA, at the origin of replication. The proposed mechanism of the succession of events involved in inhibition of plasmid DNA replication is shown in Figure 2.

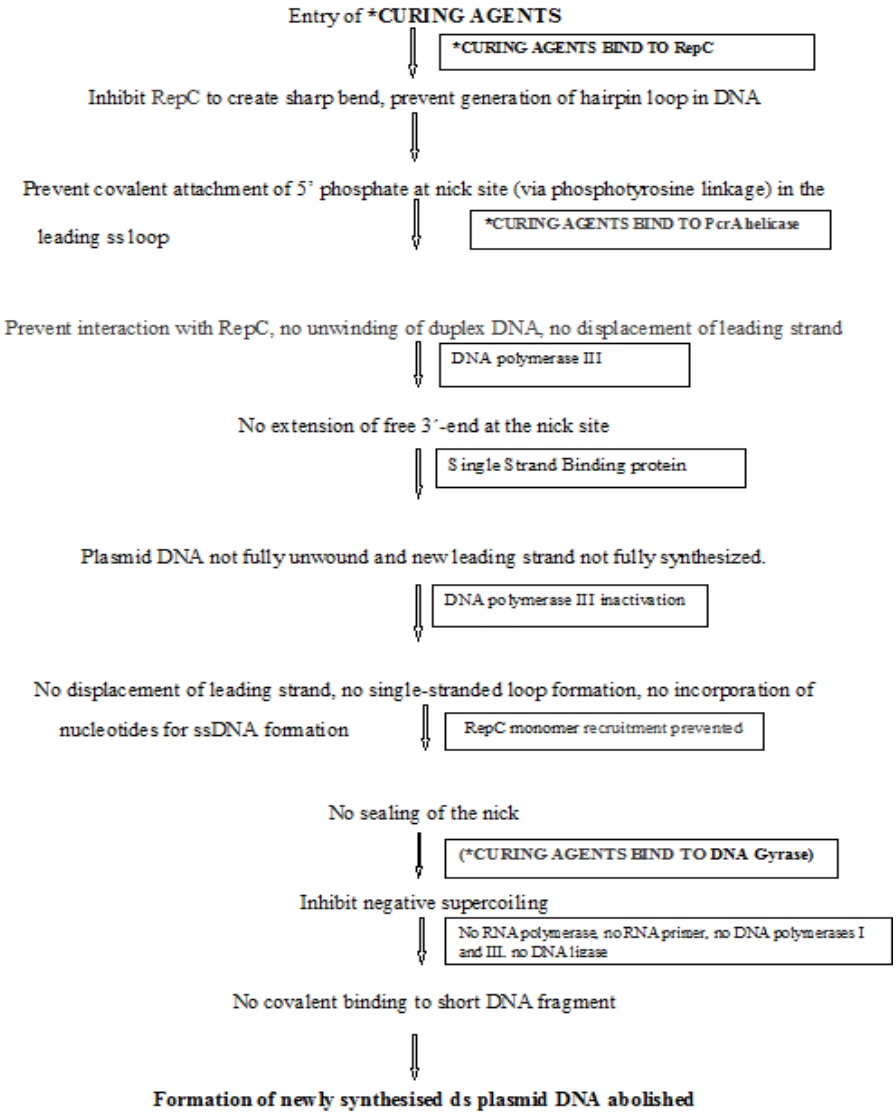

Figure 2: Steps Involved during the Action of Curing Agents with Plasmid DNA Replication

This might prevent the nick formation of a (+) strand of DNA, hindering the direct binding of Rep to the plasmid double-stranded origin, and couldn't remain covalently bound to its substrate 5'-end via a phosphotyrosine linkage. RepC have very weak nicking and relaxation activities, and it may have a little or no impact on the primary regulation of replication initiation and probably lacks replication activity of the plasmid life cycle.

Another possibility could be the binding ability of curing agents to both RepC protein and/or PcrA helicase. This might preclude the unwinding and translocation of single-stranded DNA in the resistance plasmid. Thus, multiple factors

are likely to contribute to inhibiting the broad host-range replication of RC plasmids. These factors include curing agent-RepC and/or-PcrA interactions, the inability of the RepC-PcrA complex to recruit host replication proteins, non-functionality of the lagging strand origins, and differences in the expression levels of plasmid replication genes in different organisms. Thus plasmid replication gets halted and remains functionless.

DNA Gyrase Involvement

Supercoiling mostly by DNA gyrase is known to be critical for plasmid DNA replication initiation. Obviously, DNA gyrase is required for the formation of a partial complex in contact with the cell membrane (Reyes-Domínguez et al. 2003). The presence of tricyclic drugs promoted the relaxation of plasmid DNA, by interfering with the supercoiling activity of DNA gyrase causing an end in plasmid replication (Molnár et al. 1995). Therefore, we suggest that the curing agents might form adducts with DNA gyrase, interfere with energy dependent gyrase activity and might prevent the negative supercoiling of plasmid DNA, resulting in cessation in the R-plasmid gene expression. Further, the inhibition of DNA gyrase activity by curing agents might also disturb the pre-existing gyrase in the stationary-phase cells.

The anti-plasmid action of the curing compounds might depend on the chemical structure having a planar ring system, its permeability to bacterial cell membrane might exhibit its initial binding to membrane protein DnaA thus preventing the AT-rich separation in elongation and possibly produce its effect by abolishing the covalent binding of RepC protein to the interns, inactivating PcrA helicase and DNA gyrase activity. In addition, the recruitment of DNA polymerase III, SSB proteins, RNA polymerase and the DNA ends could not be joined by DNA ligase, and no resultant DNA is supercoiled. Thus, the final inability of the host replication proteins provides non-functionality of plasmid replication genes in bacteria of periodontal disease that are directly linked to the decline in the reduction of resistant bacteria to form a biofilm.

In summary, our review is to provide insight to readers on the plasmid role in HGT, plasmid prevalence, its closeness and curing status in periodontal bacteria and the details are outlined in this article; also, the suggestive plausible plasmid curing strategies have been explored. However, detailed studies yet to be carried out in many aspects of plasmid-mediated resistance such as replacing maintenance, recombination or apparent lack of it between homologous DNA sequences, pathogenicity islands and the significance of their distinctive properties in terms of function, that could have important consequences for the more effective control of plasmid-mediated resistance in periodontal pathogens.

CONCLUSIONS

To our knowledge, this is the first presentation that discusses on traditional plasmid prevalence in periodontal pathogens, and its curing status in the same, and finally given the assumption that the plasmid curing ability could be due to 1) the planar ring system of curing agent, 2) DnaA blocking the AT-rich separation in elongation and reversing the recognition of TrfA/ TrfA like initiation proteins of plasmid, 3) prevention of the covalent binding of the Rep protein to the iterons of DNA and further PcrA helicase recruitment also abolished 4) Inhibition of DNA gyrase activity. If this speculative idea is correct, then the following questions can be addressed. What is the basis for the interaction of curing plasmid with the membrane DnaA? Is it a function of the Rep proteins, PcrA helicase, DNA gyrase, or the combination of all, or of other proteins? And, if it is either of the former possibilities, which domain binds to the membrane and which binds to the DNA? Yet to be explored!.

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ABBREVATION

AO: Acridine Orange; EtBr: Ethidium Bromide; SDS:Sodium Dodecyl Sulphate;PC:Plasmid cured; ds:double stranded; ss:Single stranded; HGT:horizontal gene transfer;

R plasmid: Resistance Plasmid ; RTF: Resistance transfer factor; RC:Rolling circular;

ESBL: Extended spectrum of β-lactams; SSB:single strand binding; PC-Plasmid Cured

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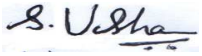

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